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(54) Wound healing composition  
prepared from amnion

(57) A preparation for use in the  
treatment of wounds is obtained from  
a culture medium in which amnion,  
preferably human amnion, has been

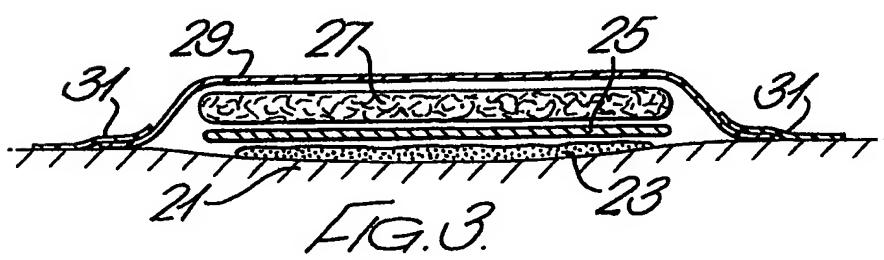
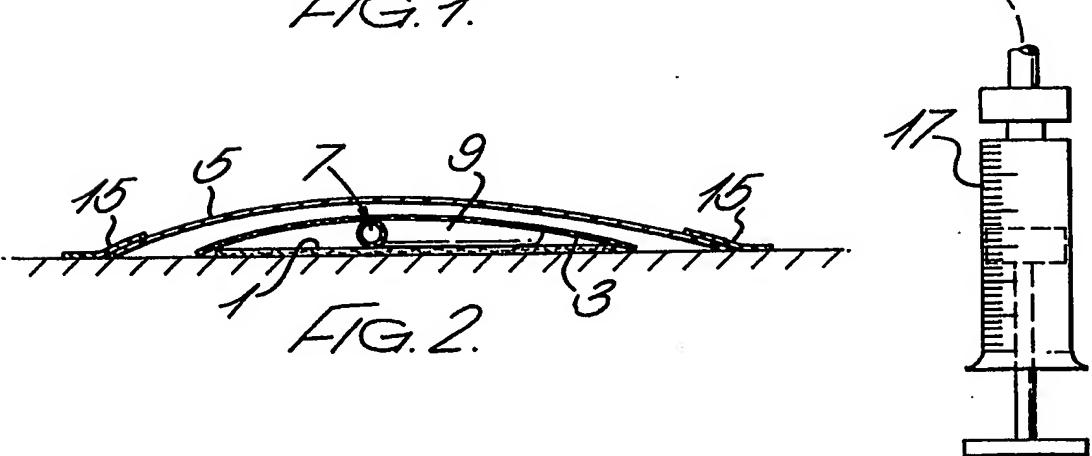
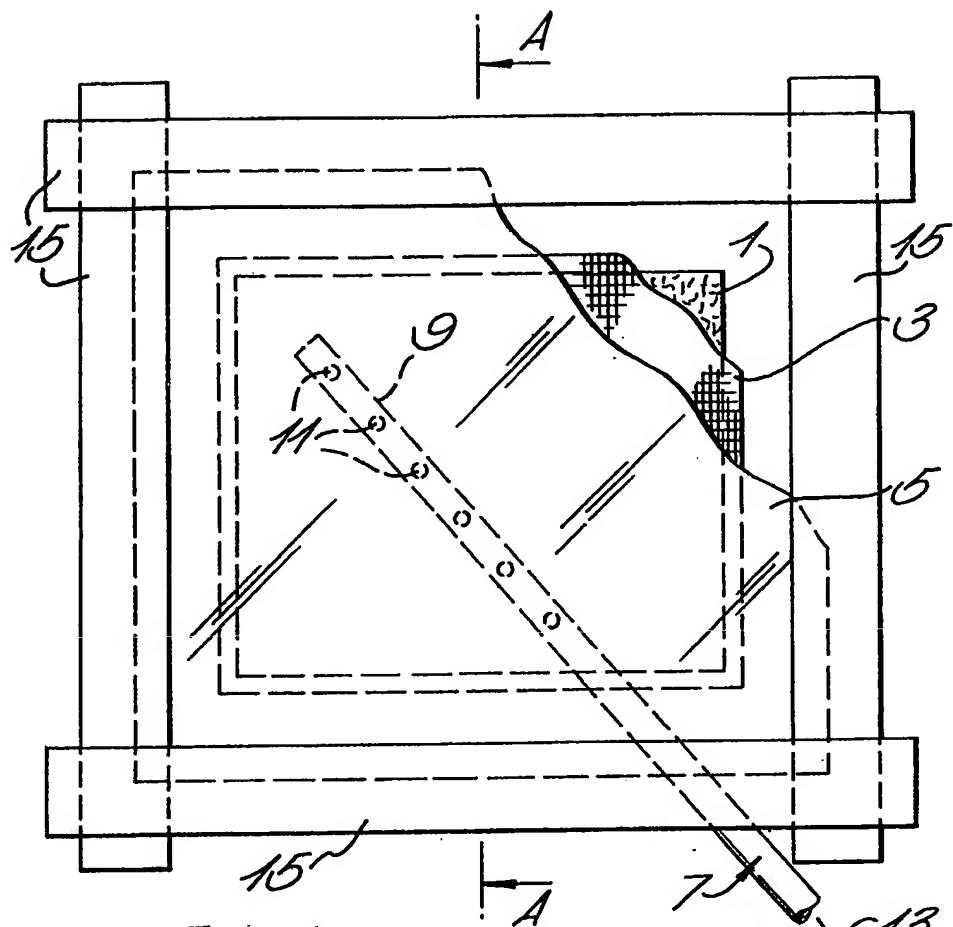
cultured. Free cells may be removed  
from the medium by centrifugation  
and, for ease and stability of storage,  
the medium may be lyophilised and  
sterilised by  $\gamma$ -irradiation.

The preparation may be used as a  
component of an ointment, or it may  
be incorporated in a surgical dressing.

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**Wound-healing composition, method of preparing it, and uses thereof**

This invention relates to compositions which are useful in the promotion of wound healing and, 5 in particular, to the preparation of such compositions from amnion, for example human amnion.

Wound healing is affected adversely by poor general health and several local factors such as 10 inadequate blood-supply and chronic infections. If direct closure of the wound is impracticable, a healthy granulating wound bed which will close either by marginal epithelialisation or by autografting is desirable. The plethora of creams, 15 powders, solutions and dressings used to promote wound healing indicates the incomplete state of the knowledge on this subject.

Human amnion has been sporadically used, since at least 1910, to promote the formation of 20 granulation tissues, and lately as a biological dressing for open wounds, including burns and chronic ulceration of the legs. For example, an article by R. N. Matthews *et al.* in British Journal of Plastic Surgery (1981) 34, 76—78 discloses the 25 use of human amnion in the treatment of a severe head burn.

A problem in the use of amnion in promoting wound healing lies in that the supply of sterile amnion is necessarily restricted, being usually 30 obtained from Caesarean deliveries. Stored and desiccated membranes have not been found to offer significant improvements over conventional sterile dressings, but when applied fresh or following preservation in normal saline, amnion 35 appears to offer advantages when compared to other types of dressings.

An article by H. Burgos and W. Page Faulk, in British Journal of Obstetrics and Gynaecology 1981, 88, 294—300 discloses a method of 40 maintaining human amnion in culture, with a view to establishing an amnion bank for subsequent therapeutic use.

Attempts have also been made to obtain cell 45 free extracts having the ability to stimulate blood vessel proliferation (i.e. angiogenesis) in model systems. For example, Tolbert *et al.* disclose in U.S. Specification No. 4,273,871 that they have investigated numerous normal human cell lines for the production of angiogenic factors, but that 50 most such cell lines possessed only poor angiogenic activity, as measured, for example, by the chorioallantoic membrane assay of Auernick *et al.*, Devel. Biol. 41, 392—394 (1974), and of Folkman, Cancer Res. 34, 2109—13 and 36, 55 110—114 (1976). Alternatively, they have found such cell lines difficult to maintain in culture.

It was found, however, that cell lines of human foreskin fibroblasts were able to synthesise the desired angiogenic factors in suitable quantities. 60 Tolbert *et al.* describe a procedure for obtaining cell-free extracts comprising such factors by harvesting cultured cells, followed by mechanically disrupting the harvested cells.

We have now found that culturing amnion in a

65 culture medium results in growth-promoting factors being released into the culture medium, and that these factors are useful as an aid to wound healing. The above-mentioned article by Burgos and Faulk nowhere suggests that used amnion growth medium, obtained as a waste product in their process, may be used in the treatment of wounds. Similarly, none of the Examples of U.S. Specification No. 4,273,871 suggests that the medium used for culturing foreskin fibroblasts may be a useful source of extracellular angiogenic factors. Indeed, it is explicitly stated that the culture medium was discarded.

According to the present invention therefore, 80 there is provided a substantially amnion-free composition for use in the treatment of wounds, comprising, or derived from, a culture medium in which amnion, preferably human amnion, has been cultured.

85 We have found amnion-free compositions according to the present invention to be surprisingly stable to lyophilisation and to prolonged storage in the lyophilised state. This stability makes such compositions particularly useful compared with intact amnion in that they can be stored conveniently and for very long periods until required. In addition, procedures for the preparation of such compositions are very much simpler than procedures involving cellular disruption, such as those described in U.S. Specification 4,273,871.

90 Preferably the amnion is in the form of sterile amniotic membrane obtained, for example, from a Caesarian delivery.

95 The amnion may be cultured in any suitable tissues culture medium, preferably buffered at a pH from 7.2 to 7.4. Examples of suitable tissue-culture media are those described by H. J. Morton, *In Vitro* 6, 89—108 (1970). Such media contain, 100 in various combinations, known essential amino acids, mineral salts, carbon sources such as carbohydrates (e.g. glucose), and vitamins.

The amnion is incubated in an atmosphere containing oxygen, and preferably also containing a relatively small concentration of carbon dioxide, which is preferably less than 5%, for example 2.5%.

110 The membrane may be cultured in one or more culture media, for example it may be cultured initially for 4 to 6 days in a first medium, such as Medium A (as described below), followed by culture in a second medium, such as Medium B (as also described below). Since contaminants from the amnion may be washed out by the culture medium during the first few days of culturing, it is preferred to use subsequent changes of culture medium for treating wounds. Similarly, when two media are used, it is the second medium which is preferred for use in accordance with the present invention.

115 After separation from the intact amnion, the culture medium is preferably subjected to centrifugation to remove free cells, and is preferably lyophilised for convenience of storage.

- The lyophilisate may be sterilised, for example by  $\gamma$ -irradiation, e.g. from a Cesium-90 source. When required for use, the lyophilisate, in the form of a dry, or substantially dry powder, may be
- 5 reconstituted by the addition of sterile distilled water.
- We have found that a preparation which is particularly active in the chorioallantoic membrane assay mentioned above may be
- 10 prepared by gel filtration chromatography of the reconstituted lyophilisate on a molecular sieve of relatively large pore-size, such as Sephadryl 300.
- According to a second aspect of the present invention, there is provided a surgical dressing comprising substantially amnion-free growth promoting-factors derived from the culture of amnion, for example a surgical dressing which has been treated with the above-mentioned
- 15 substantially amnion-free composition. Such a dressing may be impregnated with the composition in a liquid medium, and then may be dried. Alternatively, for example, the composition may be absorbed in a wound-facing layer of a surgical dressing. The dressing may also comprise
- 20 an antiseptic.
- According to a further aspect of the present invention, there is provided an ointment, for example, a water-based cream, comprising amnion-free growth promoting factors derived
- 25 from the culture of amnion. The ointment may be based on well-known recipes such as described in the British Pharmacopoeia, 1980, pages 696—702, for example emulsifying ointment, macrogol ointment, or paraffin ointment. It may also be absorbed as aqueous solution into a hydrophilic gel, based on for example, poly-2-hydroxyethyl acrylate, gelatin or the polymers described in British Patent Specification No. 1,524,899.
- 30 The ointment may also comprise other active ingredients, such as antiseptics. The inclusion of antiseptics in the ointment may reduce the dose of  $\gamma$ -radiation which is required to maintain the sterility of the made-up ointment.
- 35 The ointment may be applied directly to the wound bed and should preferably be covered with a non-adherent wound dressing such as tulle gras dressing. If the non-adherent dressing chosen is not absorbent, it may be desirable to cover it with a conventional absorbent material, such as pulped cellulose. This, in turn, may be covered with a conventional occlusive film. The occlusive film may be secured by adhesive tape.
- 40 A dressing comprising a non-adherent layer coated with ointment, an absorbent layer and an occlusive film may be pre-packed in a sterile envelope.
- 45 We also provide a method of treating a wound as an aid to healing said wound, comprising applying to the wound the substantially amnion-free composition in accordance with the present invention. The composition may for example be applied alone, or in or on a suitable carrier.
- 50 Examples of suitable carriers are the dressing and
- 55 the ointment described above. Alternatively, the

- wound may be continuously or intermittently irrigated with the composition in the form of an aqueous solution, as in the method described by Westaby *et al.* in Annals R.C.S. Eng. (1981) 63 pp. 70 353—356.
- Also provided by the present invention is a method of preparing a wound healing composition comprising culturing amnion in a culture medium, separating the medium from the amnion,
- 75 separating the medium from any free amnion cells and/or lyophilising the medium.
- A composition according to the present invention, and a method for making the same will now be described by way of example.
- 80 EXAMPLE
- Human amnions were collected aseptically from elective Caesarean sections in normal women at term. The membranes were transported in ice-cold minimal essential medium with Hank's salts supplemented with 5% foetal calf serum (FCS) or newly born calf serum (NBCS) buffered to pH 7.4 with HEPES and arrived in the laboratory 1 to 2 hours after collection. These were immediately washed in four changes of
- 85 Dulbecco's phosphate buffered saline (PBS), pH 7.35 and cleaned of blood clots and decidua remaining on the chorionic surface by means of gauze-swabs and lung-grasping forceps. Gentle handling prevented the detachment of amnion
- 90 from the attaching chorion. Large pieces of amnion (between 120 x 120 mm and 240 x 240 mm) were cut with scissors and spread, chorion side down into
- 95 243 x 243 x 18 mm polystyrene dishes, leaving the chorion-supported amnion in 150 to 200 ml of culture medium. Tissue culture medium 199 with Hank's salts supplemented with 10 per cent FCS or NBCS, buffered to pH 7.2 to 7.4 with 4 mM sodium bicarbonate and 20 mM HEPES
- 100 (Medium A) was used for the first 4 to 6 days and this was subsequently changed to Earle's salts with 10 mM sodium bicarbonate and 20 mM HEPES (Medium B). Antibiotics were added (penicillin G 100 units/ml and streptomycin 100  $\mu$ g/ml) and the cultures were incubated at 37°C in a humid atmosphere (98 per cent humidity) of 0.5 per cent and 2.5 per cent CO<sub>2</sub> in air for medium containing Hank's and Earle's salts, respectively. These CO<sub>2</sub> concentrations were adequate to
- 105 maintain the media at pH 7.2—7.4. The medium was changed after 24 hours and then two or three times a week.
- The removed medium B was pooled and then centrifuged at 30,000 g at 4°C to remove any free amnion cells. The clear supernatant was then lyophilised, and sterilised by  $\gamma$ -irradiation from a Cesium-90 source.
- 110 The lyophilisate was stored at —20°C until required for use, when it was reconstituted by addition of sterile distilled water.
- 115 A method of treatment of wound according to the present invention and surgical dressings for use in such treatment of wounds, will now be described, by way of example, with reference to
- 120

the accompanying drawings in which:—

Fig. 1 is a plan view of a dressing,

Fig. 2 is a section on line A—A of Fig. 1, and

Fig. 3 is a sectional view of an alternative

5 dressing.

Referring to the drawings, a layer of tulle gras 1 was laid over the ulcer bed of each of a number of patients suffering from chronic ulcers. A layer of surgical gauze 3 saturated with reconstituted 10 lyophilisate was laid over the tulle gras 1 and then the gauze 3, together with a small area of surrounding tissue, was covered with a plastics sheet 5. A tube 7, having an end portion 9 provided with a number of perforations 11, was 15 introduced between the tulle gras 1 and the gauze 3. The free end 13 of the tube 7 was left protruding from the edge of the plastics sheet. The edge of the plastics sheet was then fixed to the patient's skin using adhesive tape 15, in order to 20 form a fluid-tight seal between the plastics and the skin. In this way, the ulcer was enclosed in an envelope to which fluid access was available only via the tube 7. To start the treatment, 50 ml of reconstituted lyophilisate was introduced into the 25 interior of the envelope by means of a syringe 17 attached to the tube 7, after which the tube was clamped. Every 6 hours for the first 48 hours and every 12 hours for three days thereafter, the contents of the envelope were removed by 30 syringe, and replaced by 50 ml of fresh reconstituted lyophilisate.

A control group of patients was treated in identical fashion, except that the reconstituted lyophilisate was prepared from Medium B which has been incubated in the absence of amnion.

On removal of the dressings of patients treated with amnion-conditioned medium, a vascular neogenic (or granulation) response was observed. A successful auto-graft was then performed on 40 each such patient.

In contrast, the ulcers of the control patients were found to lack sufficient granulation tissue to support a successful autograft.

A dressing for use in administering a wound-healing composition in the form of an ointment is shown in Fig. 3. A wound bed 21 is covered with ointment 23 comprising the reconstituted lyophilisate described above in a slow-release matrix. A non-adherent dressing 25 is laid over the 45 ointment 23, and the non-adherent dressing is, in turn, covered with a layer of absorbent cellulose pulp 27. The entire dressing is protected from invasion by infectious agents by means of an occlusive, plastics film 29 which is attached to the 50 patient's skin using adhesive tape 31.

#### CLAIMS

1. A substantially amnion-free preparation for use in the treatment of wounds, comprising or derived from a culture medium in which amnion 60 has been cultured.

2. A preparation according to claim 1 wherein the amnion is human amnion.

3. A preparation according to claim 1 or claim 2 wherein said culture medium has been subjected to centrifugation to remove free cells.

65 4. A preparation according to any preceding claim comprising said culture medium in a lyophilised state.

5. A preparation according to claim 4 which has 70 been sterilised by  $\gamma$ -irradiation.

6. A preparation according to any preceding claim, the active fraction of which has been subjected to purification by gel-filtration chromatography.

75 7. A preparation according to any preceding claim, the culture medium having been buffered during the culture of said amnion at a pH from 7.2 to 7.4.

8. A preparation according to any preceding 80 claim, the culture medium having contained carbon dioxide at a concentration of less than 5% by weight during the culture of said amnion.

9. A preparation according to claim 8, said culture medium having contained carbon dioxide at a concentration of substantially 2.5% by weight.

10. A preparation according to any preceding claim wherein the amnion has previously been cultured in a preliminary culture medium for 4 to 6 days, and said preparation consists of, or is derived from a subsequent culture medium.

95 11. A preparation according to claim 10, wherein said preliminary culture medium is medium A as hereinbefore described, and said subsequent culture medium is medium B as hereinbefore described.

12. A surgical dressing including a preparation according to any preceding claim.

13. A dressing according to claim 12 which has been impregnated with the preparation in liquid form and then dried.

100 14. A dressing according to claim 12 wherein the preparation is absorbed in a wound-facing layer of the dressing.

15. A dressing according to any of claims 12 to 105 14 which also includes an antimicrobial agent.

16. An ointment comprising a preparation according to any of claims 1 to 11.

110 17. An ointment according to claim 16 which ointment is an emulsifying ointment, a macrogol ointment or a paraffin ointment, or is absorbed as an aqueous solution into a hydrophilic gel.

18. An ointment according to claim 16 or 17 which also comprises one or more other active ingredients.

115 19. An ointment according to claim 18 wherein said one or more other active ingredients consists of or includes an antimicrobial agent.

20. A method of preparing a wound healing preparation comprising culturing amnion in a culture medium, separating the medium from the amnion, and then separating the medium from any free amnion cells and/or lyophilising the medium.

120 21. A method of preparing a wound healing preparation, substantially as hereinbefore described with reference to the Example.

22. A substantially amnion-free preparation for

use in the treatment of wounds, substantially as hereinbefore described with reference to the Example.

23. A surgical dressing according to claim 12  
5 substantially as hereinbefore described with reference to the accompanying drawings.

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